MycoKeys 37: 1–17 (2018)
doi: 10.3897/mycokeys.37.27265
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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Tzeananiaceae, a new pleosporalean family associated with *Ophiocordyceps macroacicularis* fruiting bodies in Taiwan

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Academic editor: *T. Lumbsch* | Received 7 June 2018 | Accepted 15 July 2018 | Published 26 July 2018

Citation: Ariyawansa HA, Phillips AJL, Chuang W-Y, Tsai I (2018) Tzeananiaceae, a new pleosporalean family associated with *Ophiocordyceps macroacicularis* fruiting bodies in Taiwan. MycoKeys 37: 1–17. https://doi.org/10.3897/mycokeys.37.27265

Abstract

The order Pleosporales comprises a miscellaneous group of fungi and is considered to be the largest order of the class Dothideomycetes. The circumscription of Pleosporales has undergone numerous changes in recent years due to the addition of large numbers of families reported from various habitats and with a large amount of morphological variation. Many asexual genera have been reported in Pleosporales and can be either hyphomycetes or coelomycetes. *Phoma*-like taxa are common and have been shown to be polyphyletic within the order and allied with several sexual genera. During the exploration of biodiversity of pleosporalean fungi in Taiwan, a fungal strain was isolated from mycelium growing on the fruiting body of an *Ophiocordyceps* species. Fruiting structures that developed on PDA were morphologically similar to *Phoma* and its relatives in having pycnidial conidiomata with hyaline conidia. The fungus is characterised by holoblastic, cylindrical, aseptate conidiogenous cells and cylindrical, hyaline, aseptate, guttulated, thin-walled conidia. Phylogenetic analysis based on six genes, ITS, LSU, *rpb2*, SSU, *tef1* and *tub2*, produced a phylogenetic tree with the newly generated sequences grouping in a distinct clade separate from all of the known families. Therefore, a new pleosporalean family Tzeananiaceae is established to accommodate the monotypic genus *Tzeanania* and the species *T. taiwanensis* in Pleosporales, Dothideomycetes. The *Ophiocordyceps* species was identified as *O. macroacicularis* and this is a new record in Taiwan.

Keywords

Entomopathogenic fungi, Dothideomycetes, Multi-gene analysis, *Phoma*-like, Pleosporineae

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Introduction

We have been studying the families of Pleosporales considering both morphology and molecular phylogeny with the aim of providing a natural classification of this large order (Zhang et al. 2012, Hyde et al. 2013, Ariyawansa et al. 2013, 2014, 2015). *Phoma*-like asexual morphs have been shown to be scattered within the Pleosporineae, Pleosporales (Chen et al. 2017, Valenzuela-Lopez et al. 2018). While trying to resolve the natural classification of *Phoma*-like species in Pleosporales, several new families have been introduced within the sub-order Pleosporineae by various authors (Zhang et al. 2009, 2012, Hyde et al. 2013, Ariyawansa et al. 2015, Hernández-Restrepo et al. 2017, Valenzuela-Lopez et al. 2018).

The Pleosporales is considered to be the largest and the most diverse order of the class Dothideomycetes, comprising over 4700 species classified in 53 families (Hyde et al. 2013, Ariyawansa et al. 2015, Hernández-Restrepo et al. 2017, Valenzuela-Lopez et al. 2018). Pleosporalean species are characterised by pseudothecial ascomata usually with a papilla and a peridium composed of several layers of cells (Zhang et al. 2009, 2012, Hyde et al. 2013, Jaklitsch and Voglmayr 2016, Jaklitsch et al. 2017). Asci are bitunicate, usually fissitunicate and produced within a persistent hamathecium with or without pseudoparaphyses (Ariyawansa et al. 2013, 2014, 2015, Hyde et al. 2013). Ascospores are generally septate but vary in colour and shape, with or without a gelatinous sheath (Zhang et al. 2009, 2012, Hyde et al. 2013, Jaklitsch and Voglmayr 2016, Jaklitsch et al. 2017). Asexual morphs can be coelomycetous or hyphomycetous (Zhang et al. 2009, 2012, Hyde et al. 2013, Ariyawansa et al. 2014, 2015, Hernández-Restrepo et al. 2017, Valenzuela-Lopez et al. 2018). Members of Pleosporales are ubiquitous, occurring in various habitats and can be recognised as epiphytes, endophytes or parasites of living leaves or stems, hyperparasites on fungi or insects, lichenised or saprobes of dead plant stems, leaves or bark (Zhang et al. 2012, Hyde et al. 2013, Ariyawansa et al. 2014).

Pleosporales comprises the suborders Pleosporineae and Massarineae. (Zhang et al. 2009, 2012, Hyde et al. 2013). The suborder Massarineae was proposed by Zhang et al. (2009) and currently comprises 12 families (Tanaka et al. 2015). Pleosporineae contains numerous economically important plant and human pathogens and, at present, the suborder comprises 20 families (Valenzuela-Lopez et al. 2018).

Taiwan is an island located in the western Pacific Ocean and the importance of Taiwan's rich diversity of fungal species has been often stated in Asian and global studies (Tsai et al. 2018). A number of studies have been conducted to elucidate the diversity of pleosporalean fungi associated with various hosts and habitats in Taiwan (Chang and Wang 2009, Yang et al. 2016, Tennakoon et al. 2018), but they have rarely investigated species of Pleosporales associated with entomogenous fungi. During our investigation of pleosporalean taxa in Taiwan, a *Phoma*-like fungus was isolated from mycelium growing on the fruiting body of an *Ophiocordyceps* species. The objective of the present study was to determine the taxonomic status of the isolated fungus and the *Ophiocordyceps* species, considering both morphological characters and DNA sequence data.

Materials and methods

Fungal isolation

During the course of an exploration of ascomycetous fungi in Nantou County, Taiwan (24°06'20"N, 121°11'13"E) in July 2017, fungal mycelium was observed developing on a fruiting body of an unidentified *Ophiocordyceps* species. The mycelium was transferred to and spread on a Petri-dish containing 2% water agar (WA) and incubated at 25 °C. Single conidial isolates were established from sporulating conidiomata in Petri-dishes containing WA. Germinated conidia were transferred separately to plates of PDA (Ariyawansa et al. 2016 a, b).

Sample preparation and morphological observation

Morphological descriptions were made from isolates cultured on 2% potato dextrose agar (PDA; Difco). Preparations for microscopy were mounted in distilled water, observed with an Olympus BX51 microscope with differential interference contrast (DIC) illumination and at least 30 measurements per structure were noted. Voucher specimens were deposited in the herbarium of Department of Plant Pathology and Microbiology, National Taiwan University (NTUH). Living cultures are stored at the Department of Plant Pathology and Microbiology, National Taiwan University Culture Collection (NTUCC). Taxonomic descriptions and nomenclature details were deposited in MycoBank.

DNA extraction, PCR amplification and sequencing

Single conidial isolates were grown on PDA for 28 days at 25 °C in the dark. Genomic DNA was extracted from the mycelium using the Bioman Fungus Genomic DNA Extraction Kit (Bioman) following the manufacturer's protocol (BIOMAN SCIENTIFIC CO., LTD). For *Ophiocordyceps* species, single spore isolation was not successful. Therefore DNA was extracted directly from the ascomata using a DNA extraction kit (E.Z.N.A. Forensic DNA kit, D3591-01, Omega Bio-Tek) following the protocol of Ariyawansa et al. (2014).

PCR amplification was conducted in a 50 µl reaction volume containing 5–10 ng DNA, 0.8 units Taq polymerase, 1X PCR buffer, 0.2 mM d'NTP, 0.3 µM of each primer with the addition of 1.5 mM $MgCl_2$ (Ariyawansa et al. 2014). The PCR reactions for amplification of the internal transcribed spacer regions 1 and 2 flanking the 5.8S nrRNA gene (ITS) (Schoch et al. 2012), were performed under standard conditions (White et al. 1990, Stielow et al. 2010). PCR conditions for amplification of the partial SSU (Small subunit of the nrRNA gene) and LSU (Large subunit of the nrRNA gene) followed the protocol of Ariyawansa et al. (2015). Amplification of partial β-tubulin (*tub2*), *rpb2* (partial RNA polymerase II second largest subunit gene) and *tef1* (partial translation elongation factor 1-α gene) followed the procedure

of Woudenberg et al. (2013) and Ariyawansa et al. (2014). Primer sets used for these genes were as follows: ITS: ITS5/ITS4; LSU: LR0R/LR5; SSU: NS1/NS4; *tub2*: TUB4Rd/TUB4Fd (White et al. 1990, Liu et al. 1999, Sung et al. 2007) *tef1*: EF1-728F/ EF1-986R (Carbone and Kohn 1999) and *rpb2*: fRPB2-SF/ fRPB2-7cR (Woudenberg et al. 2013). The PCR products were visualised on 1.5% agarose gels stained with SYBR-safe DNA gel stain. PCR products were purified and sequenced by Genomics, New Taipei, Taiwan. DNASTAR Lasergene SeqMan Pro v.8.1.3 was used to obtain consensus sequences from sequences produced from forward and reverse primers. Newly generated sequences were deposited at NCBI GenBank under the accession numbers provided in Suppl. material 1: Table 1.

Sequence alignment and phylogenetic analysis

Multiple sequence alignments were produced with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html). The alignments were checked visually and adjusted manually where required. Two different datasets were prepared to evaluate two phylogenies; a Pleosporales tree and a phylogeny of the genus *Ophiocordyceps*. The first tree focused on phylogenetic placement of the new family Tzeananiaceae introduced in this study in the Pleosporales and the second to determine the placement of the *Ophiocordyceps* species (NTUH 17-004) within the genus *Ophiocordyceps*. All introns and exons were aligned individually. Regions comprising various leading or trailing gaps were excluded from the ITS, LSU, *rpb2*, SSU, *tef1* and *tub2* alignments prior to tree building. All sequences obtained from GenBank and used by Hyde et al. (2013), Ariyawansa et al. (2015), Ban et al. (2015), Hernández-Restrepo et al. (2017), Wanasinghe et al. (2017), Valenzuela-Lopez et al. (2018) are listed in Suppl. material 1: Table 1. Single alignments for each locus and the combined six-gene dataset were analysed using different tree development methods.

Maximum parsimony (MP) analyses were made using PAUP v. 4.0b10 (Swofford 2002). Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Descriptive tree statistics for parsimony (Tree Length (TL), Consistency Index (CI), Retention Index (RI), Related Consistency Index (RC) and Homoplasy Index (HI)) were calculated.

Evolutionary models for each locus were determined individually using MrModeltest v. 2.3 (Nylander 2004) under the Akaike Information Criterion (AIC) implemented in both PAUP v. 4.0b10 and MrBayes v. 3.

A maximum likelihood analysis (ML) was executed at the CIPRES webportal (Miller et al. 2010) using RAxML-HPC2 on XSEDE (v 8.2.8) with default parameters and bootstrapping with 1000 replicates (Stamatakis 2014). The subsequent replicates were printed on to the best scoring tree obtained previously.

Bayesian Markov Chain Monte Carlo (MCMC) analyses were conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The number of generations was set at

10 million and the run was stopped automatically when the average standard deviation of split frequencies fell below 0.01. Trees were saved each 100 generations. MCMC heated chain was set with a "temperature" value of 0.15. The distribution of log-likelihood scores was checked with Tracer v 1.5 to determine the stationary phase for each search and to decide if extra runs were required to achieve convergence (Rambaut and Drummond 2007, Ariyawansa et al. 2015). All sampled topologies below the asymptote (20%) were discarded as part of a burn-in procedure and the remaining trees were used to calculate posterior probabilities (BP) in the majority rule consensus tree.

Phylogenetic trees and data files were viewed in MEGA v. 5 (Tamura et al. 2011), TreeView v. 1.6.6 (Page 2001) and FigTree v. 1.4 (Rambaut and Drummond 2008). ML and MP bootstrap values equal to or greater than 70% and BP equal to or greater than 0.95 are given at each node in Figs 1, 2. Nodes with a posterior probability (PP) lower than 0.95 or MP and ML bootstrap support lower than 70% were considered unresolved.

Results

Phylogeny

The data for the trees conducted in the different analyses are shown below. In the multi-gene analyses, the topologies of the trees acquired for the individual loci were checked visually to confirm that the overall tree topology of the single datasets were comparable to each other and to that of the tree obtained from the combined dataset alignment. Phylogenetic trees obtained from the combined gene analyses are supplied below (Figs 1, 2). Alignments were analysed corresponding to a single gene study of ITS, LSU, *rpb2*, SSU, *tef1* and *tub2* of the two phylogenies. Comparison of the alignment properties and nucleotide substitution models are provided in Tables 1, 2.

Phylogeny of Pleosporales

The final alignment comprised 64 strains with 4558 characters (SSU 1019, LSU 877, ITS 450, *rpb2* 1013, *tef1* 902 and *tub2* 297). The maximum parsimony dataset consisted of 4558 characters of which 3226 were constant, 271 were variable and parsimony-uninformative and 1061 characters were parsimony-informative. Kishino-Hasegawa (KH) test showed length = 4234 steps, CI = 0.466, RI = 0.593, RC = 0.277 and HI = 0.534. The MCMC analysis of the six combined genes run for 66×10^4 generations resulted in 6600 trees. The first 1320 trees, representing the burn-in phase of the analyses, were discarded, while the remaining trees were used to calculate posterior probabilities in the majority rule consensus tree.

A best scoring RAxML tree is presented in Fig. 1, with the Likelihood value of -20128.721105. Phylogenetic trees generated from ML, MP and Bayesian analyses produced trees with similar overall topology at subclass and family level relationships

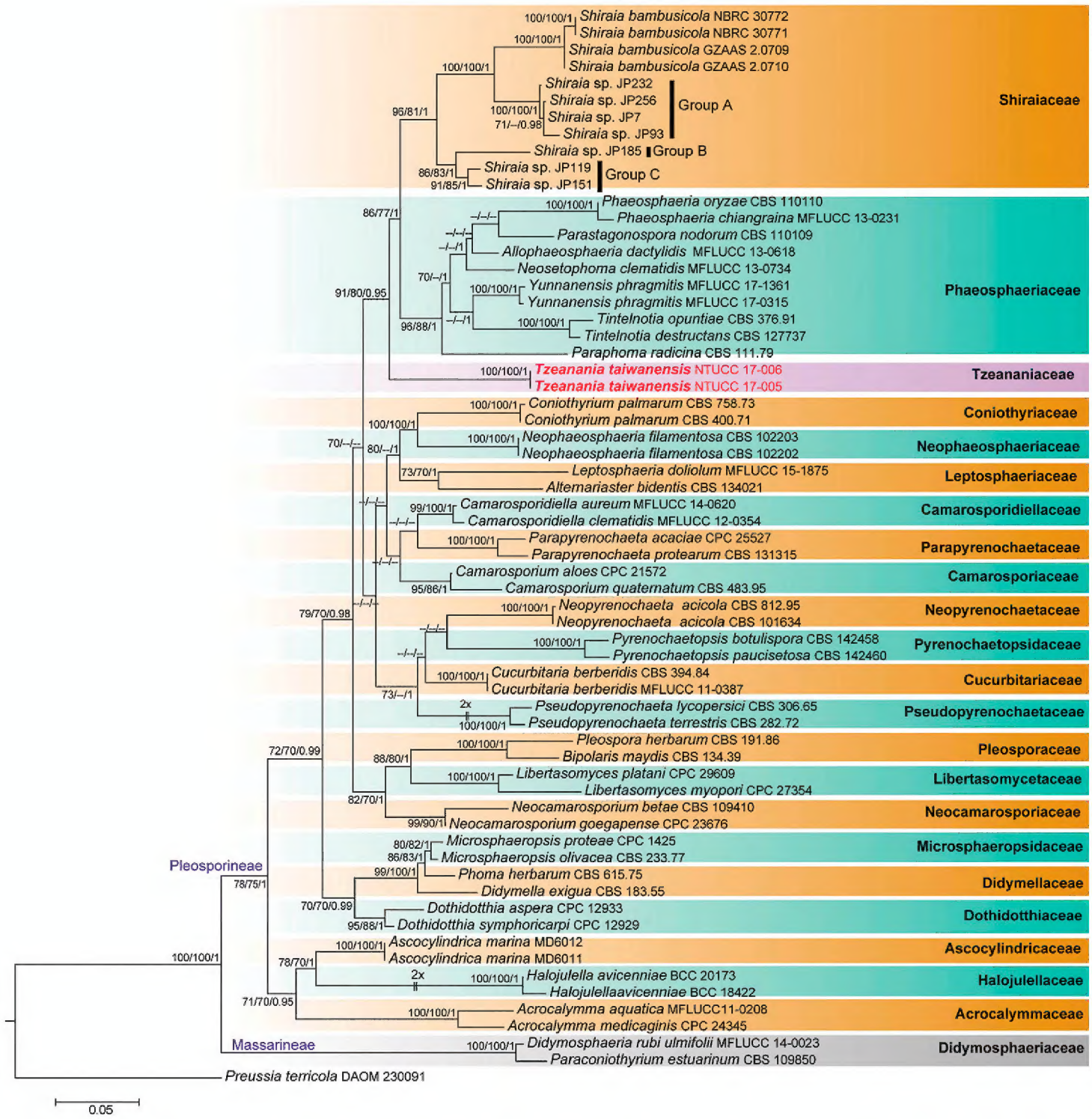

Figure 1. Phylogenetic tree (RAxML) obtained from the DNA sequence data of ITS, LSU, *rpb2*, SSU, *tef1* and *tub2* sequences of 64 strains showing taxa in suborders Massarineae and Pleosporineae within Pleosporales. The new isolates are shown in bold, red. MP and ML bootstrap values (BS) ≥70% and Bayesian posterior probabilities (PP) ≥0.95 are presented at the nodes. Several branches were shortened to facilitate presentation of the tree and this is indicated by two diagonal lines with the number of times a branch was shortened. The scale bar shows the number of estimated mutations per site. The tree was rooted to *Preussia terricola* (DAOM 230091).

Table 1. Comparison of alignment properties of genes and nucleotide substitution models used in Pleosporales phylogenetic analysis.

	LSU	SSU	*rpb2*	*tef1*	ITS	*tub2*
Alignment strategy (MAFFT v6)	G-INS-1	G-INS-1	G-INS-1 +manual	G-INS-1 +manual	G-INS-1 +manual	G-INS-1 +manual
Nucleotide substitution models for Bayesian analysis (determined by MrModeltest)	GTR+I+G	HKY+I+G	GTR+I+G	GTR+I+G	GTR+I+G	GTR+I+G

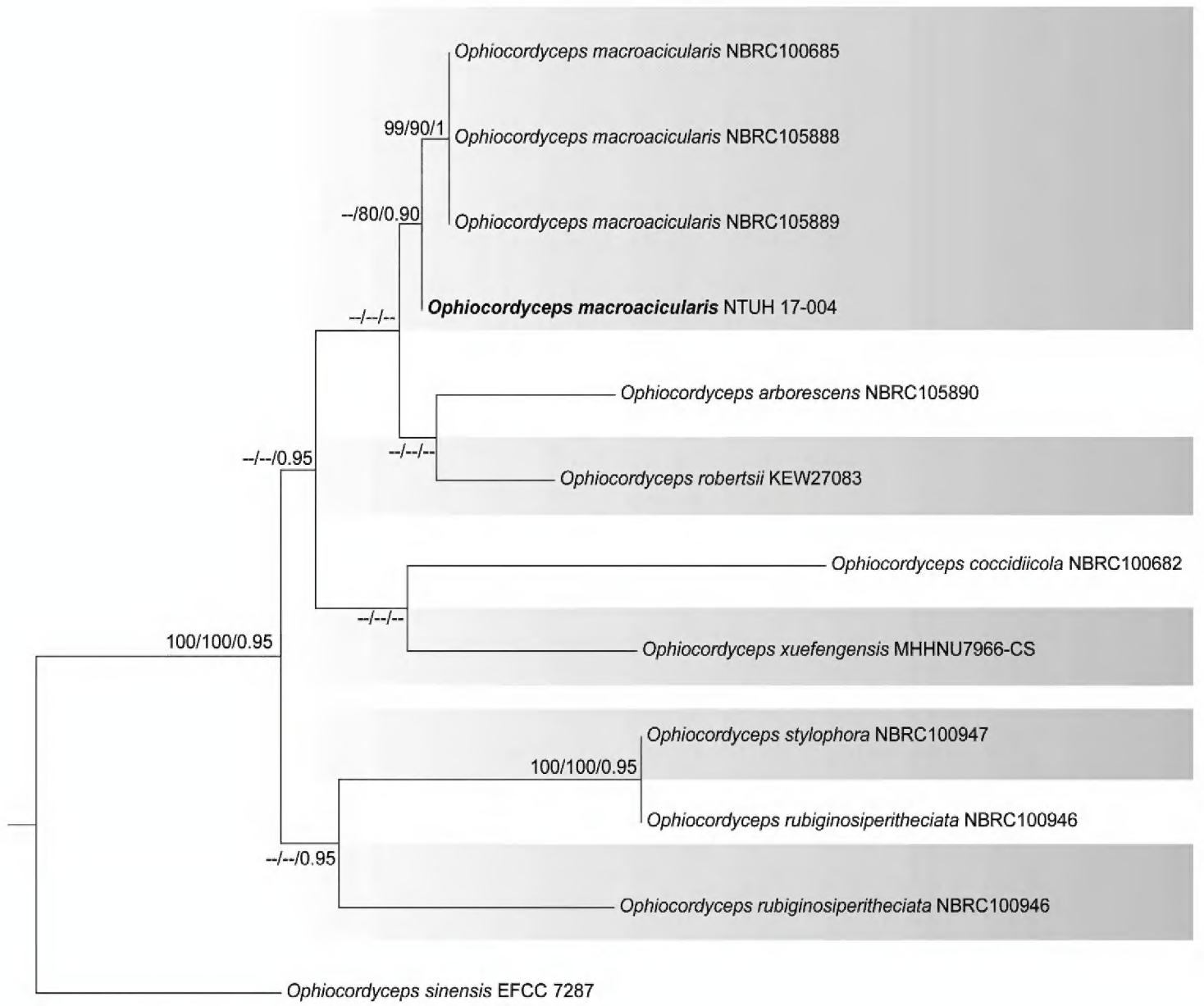

Figure 2. Phylogenetic tree (RAxML) obtained from the DNA sequence data of two loci (ITS and LSU) of *Ophiocordyceps macroacicularis* and allied taxa. The new strain is shown in bold. MP and ML bootstrap values ≥70% and Bayesian posterior probabilities ≥0.95 are presented at the nodes and the scale bar shows the number of estimated mutations per site. The tree was rooted to *Ophiocordyceps sinensis* (EFCC 7287).

Table 2. Comparison of alignment properties of genes and nucleotide substitution models used in *Ophiocordyceps* and allied species phylogenetic analysis.

	LSU	ITS
Alignment strategy (MAFFT v6)	G-INS-1	G-INS-1 +manual
Nucleotide substitution models for Bayesian analysis (determined by MrModeltest)	GTR+I	GTR+I+G

in agreement with earlier studies based on ML and Bayesian analysis (Hyde et al. 2013, Ariyawansa et al. 2015, Tanaka et al. 2015, Hernández-Restrepo et al. 2017, Wanasinghe et al. 2017, Valenzuela-Lopez et al. 2018).

The phylogenetic tree separated two distinct clades corresponding to the suborders Massarineae (represented only by the family Didymosphaeriaceae) and Pleosporineae (represented by more than 19 families). The two newly isolated strains from this study (NTUCC 17-005 and NTUCC 17-006) formed a distinct clade basal to the familial clades of *Shiraiaceae* and *Phaeosphaeriaceae* with high BS and PP support in analyses of the single locus and concatenated datasets. Hence, the novel lineage is regarded here as the new family Tzeananiaceae.

Phylogeny of *Ophiocordyceps*

The final *Ophiocordyceps* alignment comprised 12 strains. The dataset consisted of 1523 characters (LSU 899 and ITS 624). The Bayesian analysis resulted in 1×10^4 trees after 1×10^6 generations. The first 2,000 trees, showing the burn-in phase of the analyses, were discarded, while the remaining trees were used to calculate posterior probabilities in the majority rule consensus tree.

The best scoring RAxML tree is shown in Fig. 2, with the Likelihood value of -3268.294101. Phylogenetic trees acquired from ML, MP and Bayesian analysis produced trees with similar overall topology at species level relationships in agreement with a former study based on ML and Bayesian analysis (Ban et al. 2015).

Ophiocordyceps macroacicularis (NTUH 17-004), considered in this study, grouped in a well-supported clade with isolates NBRC 100685, NBRC 105888 and NBRC 105889 of *Ophiocordyceps macroacicularis* that were used by Ban et al. (2015) to introduce the species, therefore confirming the identification of the studied species.

Taxonomy

Tzeananiaceae Ariyawansa, A.J.L. Phillips & Chuang, fam. nov.
MycoBank: MB825566

Family description. *Sexual morph*: undetermined. *Asexual morph*: *Conidiomata* pycnidial, solitary or aggregated, erumpent, globose, dark brown to black. *Conidiomatal wall* of *textura angularis*. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* phialidic, hyaline, smooth-walled, ampulliform. *Conidia* hyaline, cylindrical, guttulate.

***Tzeanania* Ariyawansa, A.J.L. Phillips & Chuang, gen. nov.**
MycoBank: MB825567

Etymology. Named after the Taiwanese mycologist, Shean-Shong Tzean, in recognition of his extensive contributions towards the taxonomy of entomopathogenic fungi.

Type species. *Tzeanania taiwanensis* Ariyawansa, A.J.L. Phillips & Chuang.

Generic description. *Sexual morph*: undermined. *Asexual morph*: *Conidiomata* pycnidial, partially or entirely immersed in the agar, solitary or aggregated, erumpent, globose. *Conidiomatal wall* of *textura angularis*. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* phialidic, hyaline, smooth-walled, ampulliform. *Conidia* hyaline, smooth- and thin-walled, cylindrical, guttulate. *Chlamydospores* not observed in culture.

***Tzeanania taiwanensis* Ariyawansa, A.J.L. Phillips & Chuang, sp. nov.**
MycoBank MB825568
Fig. 3

Type. TAIWAN. Cueifong, Nantou County (24°06'20"N, 121°11'13"E), developing on a fruiting body of *Ophiocordyceps macroacicularis*, 9 July 2017, Wei-Yu Chuang, (holotype: permanently preserved in a metabolically inactive state, NTUH 17-005!; culture ex-holotype NTUCC 17-005!).

Diagnosis. Phylogeny based on ITS, LSU, *rpb2*, SSU, *tef1* and *tub2* revealed that the strains NTUCC 17-005 and NTUCC 17-006 considered in the present study formed a separate lineage sister to the familial clades of Shiraiaceae and Phaeosphaeriaceae in suborder Pleosporineae. Therefore, a new genus *Tzeanania*, a new species *T. taiwanensis* and a new family Tzeananiaceae in suborder Pleosporineae, Pleosporales are proposed here for the pycnidial coelomycete growing on the surface of the fruiting body of *Ophiocordyceps macroacicularis*.

Etymology. The epithet refers to Taiwan, where this species was collected

Description. Developing on the fruiting body of *Ophiocordyceps macroacicularis*.

Sexual morph not observed. *Asexual morph*: *Conidiomata* pycnidial, semi- or entirely immersed in the agar, solitary or aggregated, erumpent, globose, dark brown to black. *Conidiomatal wall* of *textura angularis*, 3–5 layered, composed of brown to dark brown, flattened polygonal cells. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* phialidic, hyaline, smooth-walled, ampulliform to globose, 3–5 × 0.5–2 µm, $\bar{x}$ ± SD = 4 ± 0.7 × 1.5 ± 0.3 µm. *Conidia* hyaline, smooth-walled, thin-walled, cylindrical, guttulate, 4–6 × 1–2 µm, $\bar{x}$ ± SD = 5.3 ± 0.27 × 1.5 ± 0.08 µm. *Chlamydospores* not observed in culture.

Culture characteristics. Colonies concentric circular pattern with radial furrows, entire, whitish, grey to olivaceous, with black conidiomata clustered in circular distribution; reverse concentric circular pattern with radial furrows, beige around centre and olivaceous at edge.

Distribution. Taiwan

Additional material examined. TAIWAN. Department of Plant Pathology and Microbiology, National Taiwan University, growing on a pine needles, 10 October 2017, Wei-Yu Chuang, (paratype: NTUH 17-006!, culture ex-paratype NTUCC 17-006!).

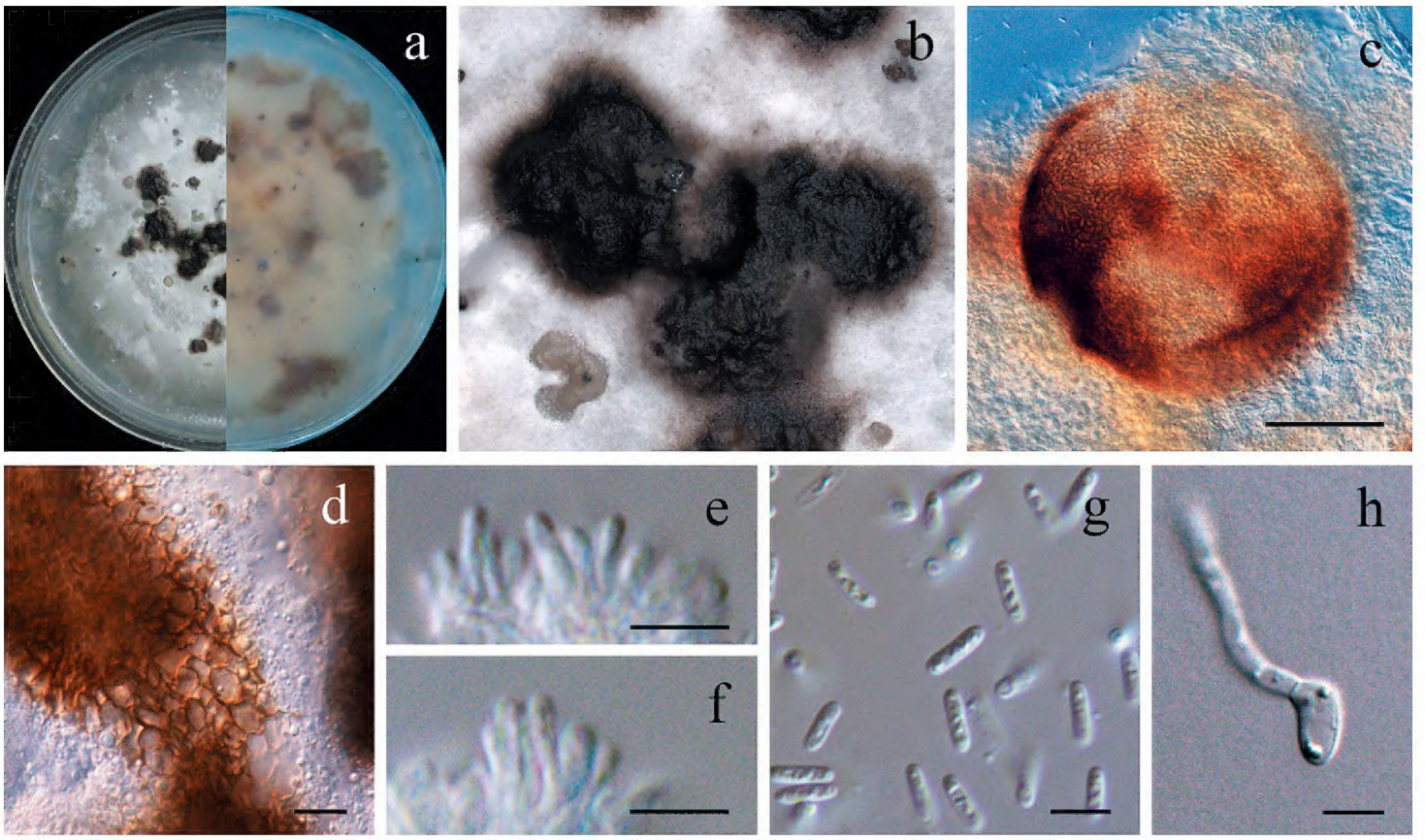

Figure 3. Morphology of *Tzeanania taiwanensis* (NTUCC 17-005) **a** Surface and lower view of colonies on PDA **b** Conidiomata sporulating on PDA **c** close-up of conidioma **d** close-up of Conidiomatal wall. **e–f** Conidiogenous cells **g** Conidia **h** Germinating conidia. Scale bars: 50µm (**c**), 10µm (**d**), 5µm (**e–h**).

Notes. *Tzeanania taiwanensis* differs from the familial type of Phaeosphaeriaceae, *Phaeosphaeria oryzae* in having erumpent, globose conidiomata, conidiomatal wall 3–5 layered, with cylindrical, aseptate, hyaline conidiogenous cells and cylindrical, hyaline, aseptate, guttulated, thin-walled conidia. *Phaeosphaeria oryzae* has immersed, uni- to multi-loculate, globose to subglobose conidiomata, conidiomatal walls comprising brown pseudoparenchymatous cells, with flattened ampulliform to doliiform, hyaline to pale brown conidiogenous cells and oblong to cylindrical, pale brown to brown, septate, smooth-walled guttulate conidia (Hyde et al. 2013).

Morphologically, *Tzeanania taiwanensis* differs from the familial type of Shiraiaceae, *Shiraia bambusicola* in having aseptate conidiogenous cells and cylindrical, hyaline, aseptate, guttulated, thin-walled conidia. *Shiraia bambusicola* has septate conidiogenous cells producing fusiform, muriform, hyaline to light brown, thick-walled conidia with irregularly arranged transverse and longitudinal septa (Hyde et al. 2013). Furthermore, *Tzeanania taiwanensis* can be clearly differentiated from *Shiraia bambusicola* by the host (*Ophiocordyceps macroacicularis* versus Bamboo) and the distribution (Taiwan versus Japan and China).

Discussion

In this study, a new family Tzeananiaceae is formally proposed in Pleosporineae, Pleosporales. This fungus was found on the surface of the fruiting bodies of *Ophiocordyceps*

macroacicularis. Phylogenetic analyses, based on DNA sequence data of ITS, LSU, *rpb2*, SSU, *tef1* and *tub2*, revealed it to form a separate lineage from all other families of Pleosporales. *Ophiocordyceps macroacicularis* is reported for the first time from Taiwan. Moreover, our study expands the base of information regarding the diversity of pleosporalean fungi associated with entomogenous taxa in Taiwan.

Molecular data play a crucial part in present-day fungal systematics, but have some limitations (Ariyawansa et al. 2014, 2015, Hyde et al. 2014, Schoch et al. 2014, Thambugala et al. 2015). The most noteworthy and disconcerting question is that the phylogeny inferred from any one gene may not disclose the evolution history of the organism (Uilenberg et al. 2004). Taylor et al. (2000) proposed operational principles for Avise and Ball's (1990) genealogical concordance species concept mainly for fungal taxa recognition. This Genealogical Concordance Phylogenetic Species Recognition (GCPSR) emphasised that species should be recognised based on genealogical concordance or genealogical non-discordance (Taylor et al. 2000, Dettman et al. 2003). This approach has been used to delineate species in several fungal groups (Udayanga et al. 2014, Manamgoda et al. 2014, Dettman et al. 2003, Ariyawansa et al. 2015). It is therefore better to integrate a polyphasic taxonomy with genotypic and phenotypic data in all forthcoming investigations (Uilenberg et al. 2004, Ariyawansa et al. 2014, 2015, Udayanga et al. 2014).

The family Shiraiaceae was introduced by Liu et al. (2013) to accommodate the bamboo parasitic genus *Shiraia* in suborder Pleosporineae. Phylogenetically, Shiraiaceae has close affinity with Phaeosphaeriaceae. Shiraiaceae species are mainly characterised by pinkish ascostromata that form on bamboo with many locules containing bitunicate asci each with six symmetrical, muriform ascospores (Hyde et al. 2013, Liu et al. 2013). The asexual morph is produced in immature ascostromata and form hyaline muriform, asymmetrical conidia (Hyde et al. 2013, Liu et al. 2013). *Shiraia* was introduced by Hennings (1900), based on *S. bambusicola*, as a monotypic genus. Later, Morakotkarn et al. (2008) reported several *Shiraia*-like strains, obtained from bamboo tissues as endophytes, which showed a close phylogenetic affinity to *Shiraia bambusicola*.

Phaeosphaeriaceae is one of the largest families in suborder Pleosporineae and includes economically important phytopathogens (Hyde et al. 2013). Species may also be found as endophytes or saprobes on different plant hosts, mainly on monocotyledons and several taxa have also been described on dicotyledons (Hyde et al. 2013). Members of Phaeosphaeriaceae are cosmopolitan and thus have been recorded from various regions around the world (Hyde et al. 2013).

Phylogenetically, *Tzeanania* has close affinity with Shiraiaceae and Phaeosphaeriaceae. To clarify the phylogeny of *Shiraia*-like fungal isolates, Morakotkarn et al. (2008) conducted a multi-gene phylogeny based on ITS, LSU and *tub2* and found three distinctive lineages, sister to *Shiraia bambusicola* clade, which were also identified with *Phoma*-like asexual morphs. Furthermore, Morakotkarn et al. (2008) concluded that *Shiraia*-like fungi Group A (Fig. 1) can be recognised as a novel species that could be allocated into a novel genus/species related to *S. bambusicola*. Single gene analysis

Figure 4. Morphology of *Ophiocordyceps macroacicularis* (NTUH 17-004) **a** Close-up of ascomata **b** Close-up of the peridium **c** Hyaline, cylindrical, eight-spored ascus **d** Needle-shaped, multi-septate, hyaline ascospores. Scale bars: 20 μm (**b**), 50 μm (**c–d**).

of LSU and SSU showed that our strains formed a basal lineage to the familial clade of the Shiraiaceae. Therefore to confirm phylogenetic affinity of our isolates with *S. bambusicola* and *Shiraia*-like fungi groups A, B and C, we additionally conducted a comprehensive phylogeny derived from 3 genes LSU, ITS and TUB (data not shown). We produced a tree with similar topology to the one reported by Morakotkarn et al. (2008) while our new strains formed a distinct lineage sister to the familial clades of Shiraiaceae and Phaeosphaeriaceae, which further confirms the uniqueness of the new family Tzeananiaceae in suborder Pleosporineae.

Ophiocordyceps macroacicularis S. Ban et al. was introduced by Ban et al. (2015) and was recently recorded from Thailand by Luangsa-ard et al. (2018) based on molecular phylogeny together with morphology (Figs 2, 4). To the best of our knowledge, this is the first record of *O. macroacicularis* in Taiwan. Sun et al. (2016) introduced a hyphomycetous taxon, *Calcarisporium cordycipitiicola*, which was also found to infect the fruiting bodies of *Cordyceps militaris* causing significant quality and yield losses. Even though we were able to obtain a single spore culture of *T. taiwanensis* (NTUCC 17-006) using the fruiting structures formed on PDA (Fig. 3b), single spore isolation of *O. macroacicularis* was not successful. Therefore, we could not clarify the exact nutritional mode of *T. taiwanensis* or its interaction with *O. macroacicularis*. Therefore, further studies are essential to understand the interaction between this unusual fungus and its host.

Acknowledgements

This study was funded by the Ministry of Science and Technology, Taiwan (MOST project ID: 106-2621-B-002-005-MY2). Alan J.L. Phillips acknowledges the support from Biosystems and Integrative Sciences Institute (BioISI, FCT/UID/ Multi/04046/2013). We appreciate the support given by Professors CP Lin, WC Shen, TL Shen, RF Liou, Associate Professor CL Chung and Chia-Ming Hu. H. A. Ariyawansa is grateful to A.D Ariyawansa, D.M.K Ariyawansa, Ruwini Ariyawansa and Amila Gunasekara for their valuable suggestions.

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